Autologous endothelialized small-caliber vascular grafts engineered from blood-derived induced pluripotent stem cells

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Abstract: An ideal cell source for human therapeutic and disease modeling applications should be easily accessible and possess unlimited differentiation and expansion potential. Human induced pluripotent stem cells (hiPSCs) derived from peripheral blood mononuclear cells (PBMCs) represent a promising source given their ease of harvest and their pluripotent nature. Previous studies have demonstrated the feasibility of using PBMC-derived hiPSCs for vascular tissue engineering. However, so far, no endothelialization of hiPSC-derived tissue engineered vascular grafts (TEVGs) based on fully biodegradable polymers without xenogenic matrix components has been shown. In this study, we have generated hiPSCs from PBMCs and differentiated them into SMA- and calponin-positive smooth muscle cells (SMCs) as well as endothelial cells (ECs) positive for CD31, vWF and eNOS. Both cell types were co-seeded on PGA-P4HB starter matrices and cultured under static or dynamic conditions to induce tissue formation in vitro. The resulting small diameter vascular grafts showed abundant amounts of extracellular matrix, containing a thin luminal layer of vWF-positive cells and a subendothelial SMA-positive layer approximating the architecture of native vessels. Our results demonstrate the successful generation of TEVGs based on SMCs and ECs differentiated from PBMC-derived hiPSC combined with a biodegradable polymer. These results pave the way for developing autologous PBMC-derived hiPSC-based vascular constructs for therapeutic applications or disease modeling. STATEMENT OF SIGNIFICANCE: We report for the first time the possibility to employ human peripheral blood mononuclear cell (PBMC)-derived iPSCs to generate biodegradable polymer-based tissue engineered vascular grafts (TEVG), which mimic the native layered architecture of blood vessels. hiPSCs from PBMCs were differentiated into smooth muscle cells as well as endothelial cells. These cells were co-seeded on a biodegradable PGA/P4HB scaffold and cultured in a bioreactor to induce tissue formation in vitro. The resulting small diameter TEVG showed abundant amounts of extracellular matrix, containing a SMA-positive layer in the interstitium and a thin luminal layer of vWF-positive endothelial cells approximating the architecture of native vessels. Our findings improving the generation of autologous vascular replacements using blood as an easily accessible cell source.

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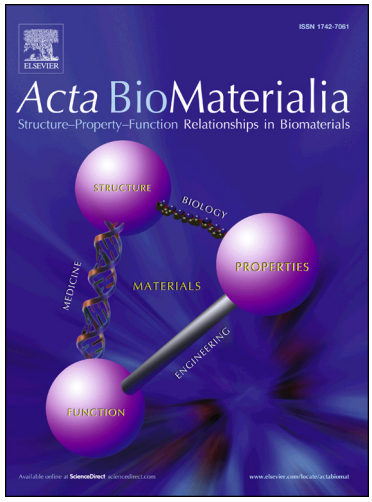

Full length article

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Autologous endothelialized small-caliber vascular grafts engineered from blood-derived induced pluripotent stem cells

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Running title:

Blood cell-derived hiPSCs for engineered vascular grafts

Abstract

An ideal cell source for human therapeutic and disease modeling applications should be easily accessible and possess unlimited differentiation and expansion potential. Human induced pluripotent stem cells (hiPSCs) derived from peripheral blood mononuclear cells (PBMCs) represent a promising source given their ease of harvest and their pluripotent nature. Previous studies have demonstrated the feasibility of using PBMC-derived hiPSCs for vascular tissue engineering. However, so far, no endothelialization of hiPSC-derived tissue engineered vascular grafts (TEVGs) based on fully biodegradable polymers without xenogenic matrix components has been shown.

In this study, we have generated hiPSCs from PBMCs and differentiated them into αSMA- and calponin-positive smooth muscle cells (SMCs) as well as endothelial cells (ECs) positive for CD31, vWF and eNOS. Both cell types were co-seeded on PGA-P4HB starter matrices and cultured under static or dynamic conditions to induce tissue formation *in vitro*. The resulting small diameter vascular grafts showed abundant amounts of extracellular matrix, containing a thin luminal layer of vWF-positive cells and a subendothelial αSMA-positive layer approximating the architecture of native vessels. Our results demonstrate the successful generation of TEVGs based on SMCs and ECs differentiated from PBMC-derived hiPSC combined with a biodegradable polymer. These results pave the way for developing autologous PBMC-derived hiPSC-based vascular constructs for therapeutic applications or disease modeling.

Keywords:

Peripheral blood mononuclear cells, induced pluripotent stem cells, vascular tissue engineering, blood vessels, biodegradable polymer

1 Introduction

Cardiovascular disease remains the leading cause of morbidity and mortality, accounting for more than 17 million deaths per year worldwide [1]. Replacement of affected vascular tissues has been widely used to treat structural cardiovascular disease such as coronary heart disease, aortic aneurysm and peripheral vascular disease [2]. However, successful treatment is often limited by the lack of suitable autologous replacement tissues. So far, expanded polytetrafluoroethylene (ePTFE), polyethylene terephthalate (PET) and polyurethane (PU) have been successfully used to produce synthetic vascular grafts with a diameter greater than 6 mm [3, 4]. Grafts smaller than 6 mm in diameter lead to many complications including acute thrombosis and stenosis caused by the lack of functional endothelium coverage [5]. Several approaches focusing on the prevention of thrombogenicity of these synthetic materials have been undertaken; in particular, coating of the luminal surface with heparin and other anticoagulant materials has been attempted - however, with limited success [6, 7]. In addition, these grafts do not show growth-adaptive behavior, which would be of particular importance in children that require multiple reoperations due to the lack of growth potential [8]. Therefore, the current synthetic vascular replacements are suboptimal and tissue engineering (TE) is proposed as a solution by replacing tissues or organs with functional autologous replacement constructs. Tissue-engineered vascular grafts (TEVGs) represent living vascular replacement constructs, where vascular cells are seeded onto a three-dimensional biodegradable scaffold and then stimulated via conditioning in a bioreactor to promote tissue formation *in vitro*. However, mature vascular cells are associated with limited access, expansion and differentiation potential [9]. In general, the source of vascular cells represents a central problem for cell-based vascular therapies as isolated vascular cells from donor tissues are limited in availability and quantity. Moreover they show limited proliferation, extra cellular matrix (ECM) formation capacity, and cellular functionality following the extensive *in vitro* expansion process [10]. Therefore, in recent years different cell sources have been tested also with a focus on their use for vascular tissue engineering [11, 12]. Smooth muscle cells (SMCs) can be differentiated out of alternative cell sources such as

bone-marrow mesenchymal stem cells [13, 14], endothelial progenitor cells [15], or adipose-derived stem cells [16]. Nonetheless, the proliferation and differentiation capabilities of adult stem cells considerably decline with aging donors [17] and strongly depends on the tissue source cells are harvested [18]. An interesting alternative is the use of the so-called human induced pluripotent stem cells (hiPSCs). hiPSCs share similar proliferation capacity, differentiation potential and gene expression with embryonic stem cells (ESCs) and are able to differentiate into any cell type of the human body. Furthermore, hiPSCs can be generated from potentially any terminally differentiated cell [19] providing an unlimited source of proliferating cells and overcoming therefore the limitations of confined donor cell availability as well as limited proliferation capacity. Thus, the use of hiPSCs for tissue engineering may represent an ideal cell source and therefore the possibility to reprogram somatic cells from a patient and generate 'customized' tissues from only few cells has created substantial hope.

Since the first iPSCs were generated from skin fibroblasts, many other sources of adult somatic cells have been tested for their suitability for reprogramming [19]. For instance, the generation of hiPSCs from peripheral blood mononuclear cells (PBMCs) has many benefits, such as their ease of harvest or direct reprogramming of harvested cells without *in vitro* propagation.

Therefore, the present study aims at the generation of TEVGs based on SMCs and endothelial cells (ECs) differentiated from PBMC-derived hiPSCs combined with a fully biodegradable polymer matrix based on PGA and P4HB. These results pave the way for developing autologous vascular replacements, as well as a basis for modeling human disease *in vitro* using blood as the only, easily accessible, cell source.

2 Materials and Methods

2.1 Isolation and reprogramming of PBMC

Human peripheral blood was collected with written informed consent according to the permission from the cantonal ethics commission of Zurich, Switzerland [KEK-ZH-2014-0430]. PBMCs were isolated according to standard protocols using the Ficoll-Paque method. The reprogramming of PBMCs into hiPSCs was induced according to previously reported protocol [20]. Shortly, PBMCs reprogramming was achieved using viral vectors, which lead to the simultaneous expression of the specific transcription factors OCT4, SOX2, c-MYC and KLF4 (STEMCCA lentiviral vector, Boston University School of Medicine, Boston, USA). The infected cells were then seeded on mitotically inactivated mouse embryonic fibroblasts (MEFs) and cultured in human iPSCs medium (DMEM/F12 (Invitrogen, Switzerland), 20% Knockout serum replacement (Invitrogen, Switzerland), 100mM nonessential amino acids (Sigma, Switzerland), 50mM 2-mercaptoethanol (Life Technologies, Switzerland), 1mM L-glutamine (Sigma, Switzerland), 50 U/ml penicillin/streptomycin (Sigma, Switzerland), 10ng/ml basic fibroblast growth factor (bFGF) (Life Technologies, Switzerland)). The reprogramming process took about 15-30 days and finally, embryonic stem like colonies were picked and expanded.

2.2 Characterization of PBMC-derived hiPSCs

Three different hiPSC lines (IREMi001A, IREMi001B, IREMi001C) were characterized using a standard series of quality controls as follows:

- **Quantitative Real-Time PCR** (qRT-PCR): Total RNA was extracted using RNeasy Mini Kits (Qiagen, Switzerland) and reverse transcription was carried out using Superscript III RT (Invitrogen, Switzerland) according to the manufacturer`s instructions. qRT-PCR were performed using Rotor-Gene SYBR Green PCR Kit SYBR (Qiagen, Switzerland) and primers for Oct4, Sox2, Nanog, Rex1, and Dppa3 (Microsynth, Switzerland). Reactions were run in triplicates and using standard

conditions on a Rotor-Gene Q (Qiagen, Switzerland). All primers are listed in Supplemental Table 1.

- **Alkaline Phosphatase** (AP) activity: Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich, Switzerland), washed with PBS and later with alkaline buffer for 10 minutes. Then alkaline phosphatase solution (10ml alkaline buffer containing 45µl NBT and 35µl BCP) was added and the reaction was stopped with TRIS-EDTA for 15 seconds. Staining were analyzed with DMIL LED fluorescence microscope (Leica, Germany) and the Leica software (Leica, Germany).
- **Immunofluorescence**: hiPSCs were cultured on a 3.5cm dish, fixed with 4% paraformaldehyde, and incubated over night at 4°C with primary antibodies (Supplemental table 2). Secondary antibodies were incubated for 1 hour (Supplemental Table 2) and picture were taken and analyzed with DMIL LED fluorescence microscope and the Leica software.
- **In-vitro embryoid body formation**: hiPSCs were cultured in suspension in hiPSC medium without bFGF to form embryoid bodies (EB). After 3 weeks EB were harvested and RNA isolated for gene expression or plated for further differentiation.
- **Karyotyping**: hiPSCs were arrested in metaphase by treatment with 80 ng/ml colcemide for 1 h. Then harvested and treated with hypotonic solution (0.075 M KCl), followed addition of fix solution (MeOH 3:1 Acetic Acid). Metaphases spreads were stained by Quinacrine (Sigma). Images were taken on a Zeiss Axioskop HBO 50 fluorescent microscope (Zeiss) and arranged in Ikaros Software (MetaSystems).
- **Teratoma assay:** $2x10^5$ undifferentiated, MEF free hiPSCs were injected subcutaneously into one dorsal flank of NOD.CB17-Prkdc^{scid} mice. 10 to 12 weeks later teratomas were microscopically (histology) analysed. The veterinary office of the Canton Zurich, Switzerland approved all animal experiments (ZH-171/2014).

180 **2.3 Differentiation of hiPSCs**

181 hiPSC were separated from MEFs and cultured in suspension for 7 days in ultra-low
182 attachment dishes (Corning, USA) in hiPSC medium without bFGF to form EBs, which were
183 then plated for further differentiation into SMCs and ECs

184

185 ***2.3.1 Smooth muscle cells***

186 For muscle differentiation, attached EBs were cultured in high glucose DMEM (Sigma,
187 Switzerland), 10% FBS (Sigma, Switzerland), 5% horse serum (Life Technologies,
188 Switzerland), 100mM nonessential amino acids, 100mM 2-mercaptoethanol, 1mM L-
189 glutamine, 50 U/ml penicillin/streptomycin. After 14 days medium was changed to Smooth
190 Muscle Growth Medium-2 (SmGM-2) (Lonza, Switzerland) and further cultured for 14 days.
191 All differentiated cells were fixed for immunofluorescence or flow cytometric analysis (see
192 2.4), lysed for total RNA extraction or further cultured for TE (antibodies and primers are
193 listed in Supplemental Tables 1 and 2). As a positive control primary human aortic SMCs
194 were commercially obtained (CC-2571; Lonza, Switzerland).

195

196 ***2.3.2 Endothelial cells***

197 The differentiation of hiPSCs into ECs was induced according to previously reported protocol
198 with some modifications [21]. Briefly, hiPSCs were cultured in suspension for 10 days in a
199 medium consisting of DMEM, 20% FBS, 2mM L-glutamine, 100uM 2-mercaptoethanol,
200 100uM NEAA, 50ng/ml ascorbic acid, 125U/ml penicillin, 125mg/ml streptomycin. EBs were
201 dissociated into single cells with 2 mg/ml collagenase B (Roche, Switzerland) for 2 hours
202 then Cell Dissociation Buffer (Invitrogen, Switzerland) for 15 min at 37°C shaking at 1,100
203 rpm. VE-cadherin+ cells were isolated by magnetic bead sorting (Miltenyi Biotec, USA).
204 Isolated hiPSC-derived ECs were plated at 20,000 cells/cm^2 on fibronectin coated dishes
205 (Corning, USA) in endothelial cell medium (EGM-2; Lonza, Switzerland). All differentiated
206 cells were either fixed for immunofluorescence or flow cytometric analysis (see 2.4), or lysed
207 for total RNA extraction or further cultured for TE (antibodies and primers are listed in

Supplemental Table 1 and 2). Moreover, as a positive control ECs were isolated from human umbilical cord with informed consent according to the cantonal ethics commission of Zurich, Switzerland [KEK-ZH-2009-0095].

2.4 Flow cytometric analysis

For the purpose of nuclear transcription factor staining, the cells were washed with PBS, fixed in 1% paraformaldehyde (*Electron Microscopy Sciences*, USA), for 20 min at RT and permeabilized by FACS buffer and 0.5% Saponin (Sigma-Aldrich, Switzerland) for 20 min at 4°C. Incubation with directly labeled αSMC antibody was carried out at 4°C for 25 min, followed by 3 washes. For the purpose of surface antigen staining, the cells were washed with PBS, incubated with the directly labeled CD31 antibody for 20 min at 4°C and subsequently washed. The antibodies listed in supplementary Table 2. Cells were acquired using a LSR Fortessa (BD Bioscience, USA) and the data sets were analyzed by FlowJo software (Tree Star, Inc., USA).

2.5 Tissue Engineering

2.5.1 Surface morphology of biomaterials

Samples were mounted on electron imagining stubs using carbon tape and a field emitting electron microscope used to image samples at 15kV power (Zeiss, FESEM Ultra Plus) for clarity. For each sample, four regions of interest (ROI) measuring 1000×800µm were imaged from which five fibers per ROI were measured using Image J software (NIH, v1.48s, line tool) to determine the average fiber diameter of an ROI. For scaffold porosity, an additional four ROIs measuring 3000×2250µm were imaged. Each porosity ROI image was then automatically thresholded in Image J and porosity was defined as the percent area that was non-fiber (ie empty/porous space).

2.5.2 Scaffold fabrication

Vessels were fabricated from non-woven polyglycolic acid (PGA) meshes (thickness 0.5 mm; specific gravity 70 mg/cm^3; Cellon, Luxembourg) and coated with 1.75% poly-4-hydroxybutyrate (P4HB; TEPHA, Inc., USA) by dipping into a tetrahydrofuran (THF) solution (Sigma-Aldrich, Switzerland). After solvent evaporation and vacuum drying overnight, the scaffolds were placed into a 70% EtOH (Sigma-Aldrich, Switzerland) for 30 min to obtain sterility, followed by two washing cycles with PBS (Sigma-Aldrich, Switzerland). Thereafter, scaffolds were pre-incubated in SmGM-2 with supplements for 12–24 hours to facilitate cell attachment.

2.5.3 Cell seeding

hiPSCs-derived SMCs were seeded onto tubular scaffolds (length 1cm and inner diameter 0.7mm) using 1.0*10^{6}cells/cm^2. Fibrinogen (Sigma-Aldrich, Switzerland) (10 mg/mL of active protein) and thrombin (Sigma-Aldrich, Switzerland) were prepared, used and titrated to an optimal clotting time of approximately 30 sec by adapting the concentration of fibrinogen. The cells were resuspended in a fibrinogen-thrombin co-solution and evenly seeded into the inner lumen and onto the outer layer. After static incubation of seeded constructs in SmGM-2 (with supplements and 0.9mM of L-ascorbic acid-2-phosphate (Sigma-Aldrich, Switzerland)) for 7 days, they were either placed into a bioreactor under pulsatile flow for additional mechanical stimulation via shear stress (dynamic condition) or kept under static conditions. The pulsatile flow was directed through the inner lumen and mimicked the native cardiovascular environment with a constant flow over time (2.5ml/min). After 21, 42 and 63 days of culture, hiPSCs-derived ECs (1.0*10^{6}cells/cm^2) were seeded into the lumen of TEVGs from both sides and rotated for 7 minutes to increase an evenly seeding. Afterwards TEVGs were kept for additional 3 days under static culture. The constructs were harvested after 24, 45 and 66 days of culture and immediately processed for immunohistochemistry and quantitative tissue analysis.

2.6 Qualitative Tissue Analyses

For immunohistochemical analysis of TEVGs, 5µm sections derived from blocks of formalin-fixed, paraffin-embedded tissue were mounted on glass slides (SuperFrost Plus, Menzel Gläser,Germany), deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E), Masson Trichrome (MT) or Elastica van Gieson (EVG) using standard histological techniques. All sections were analyzed using a Mirax Midi BF slide scanner and processed using MIRAX viewer (Zeiss, Germany). In addition, immunofluorescence for αSMA and vWF were performed by IHC-Service Lab, University Hospital Zurich, Switzerland. Images were captured with a DM6000B fluorescence microscope (Leica, Germany) and processed using the Leica software (Leica, Germany). Furthermore, each sample was analyzed using Image J software to determine the average wall thickness.

2.7 Quantitative Tissue Analysis

TEVGs (n = 4 per clone) were minced, lyophilized, and analyzed using biochemical assays for total deoxyribonucleic acid (DNA) content as an indicator for cell number, hydroxyproline (HYP) content as an indicator for collagen, as well as for glycosaminoglycan (GAG) content. All samples were digested in papain (Sigma-Aldrich, Switzerland) solution (300 µg/mL in PBS with 5mM EDTA) (Sigma-Aldrich, Switzerland) and 5mM cysteine (Sigma-Aldrich, Switzerland) at 65°C for 16 hours. For measuring the cellularity of the constructs, the DNA amount was quantified according to manufacturer's protocol (Life Technologies, Switzerland, No. P11496). The GAG content was determined using a modified version of the protocol described by Farndale et al. [22], as previously described, and a standard curve prepared from chondroitin sulfate from shark cartilage (Sigma-Aldrich, Switzerland). HYP was determined with a modified version of the protocol provided by Huszar et al. [23]. Remnants of the co-polymers matrices were visualized using polarization microscopy.

2.8 Statistical Methods

Quantitative data are presented as mean ± standard deviation. Quantitative tissue analysis were statistically evaluated using an unpaired students-t-test. P-values $p<0.05$ were considered statistically significant (*$p<0.05$, **$p<0.03$, ***$p<0.001$, ****$p<0.0001$). Post-hoc correction was performed using the Bonferroni method where applicable. Kolmogorov-Smirnov normality test was used to confirm that the datasets were normally distributed ($p>0.05$). All statistical analyses were performed using the GraphPad Prism 5 software (GraphPad Software Inc., USA).

3 Results

3.1 Characterization of human PBMCs derived iPSCs

PBMCs were isolated from human peripheral blood using the Ficoll-Paque method. Reprogramming into hiPSCs was induced according to previously reported protocols [25] by simultaneous transduction of viral vectors containing OCT4, SOX2, c-MYC and KLF4. After about 30 days, embryonic stem-like colonies started to appear. Colonies displaying the characteristic morphology of human ESCs (Figure 1A) could be picked and expanded. These colonies showed high alkaline phosphatase (AP) activity (Figure 1B) and expressed the pluripotency associated transcription factors OCT4, SOX2, NANOG, REX1, and DPPA3 (Figure 1C). All markers were upregulated compared to the original, non-reprogrammed PBMCs, suggesting their transition to a pluripotent, undifferentiated state. Furthermore, the human specific pluripotency markers TRA-1-81 and TRA-1-60 (Figure 1D and 1E) were also exclusively expressed in the hiPSC colonies. To assess the pluripotent potential of the generated hiPSCs, cells were first differentiated *in vitro* into embryoid bodies (EBs). Analysis of the expression of early differentiation markers revealed differentiation into all three germ layers: endoderm (FOXA2 and CXCR4), ectoderm (PAX6 and DCX) and mesoderm (MSX1 and PAX3) (Figure 1F). In addition, hiPSCs were injected subcutaneously into NOD-SCID mice and teratomas were generated after 10-12 weeks. Histological analyses revealed the presence of all three germ layers including ectoderm (neural epithelium), mesoderm (cartilage) and endoderm (intestinal epithelium) (Figure 1G-I). Taken together, these results confirmed the pluripotent nature of the generated hiPSCs. Three different clones were expanded and tested for the pluripotent status (Supplementary Figure 1). Each assay confirmed the transition from peripheral blood cells into undifferentiated pluripotent cells for all clones. Furthermore, karyotyping has been performed as an additional quality control and reprogrammed cell retained a normal karyotype (Supplementary Figure 2).

3.2 Characterization of human iPSC-derived smooth muscle and endothelial cells

We next investigated the ability of three generated hiPSC clones (IREMi001A, IREMi001B, IREMi001C) to differentiate into SMCs and ECs, in order to use them for vascular TE applications. Under muscle-inducing culture conditions, hiPSCs gradually lost the expression of the pluripotency genes and gained the expression of the SMCs specific genes like αSMA, calponin and SMMHC (Figure 2A and 2B). Interestingly, all hiPSC-derived SMC clones revealed positive expression of the smooth muscle myosin heavy chain (SMMHC) isotype SM2 (Supplementary Figure 4). Moreover, the hiPSC-derived SMCs showed a similar marker profile at RNA and protein level when compared to primary human aortic SMCs (Figure 2A and 2C, Supplementary Figure 4). RT-PCR and immunostaining have been performed at day 21 and day 35 after differentiation start. To evaluate the differentiation efficiency flow cytometric analysis for αSMA were performed and displayed 94.6-98.3% αSMA-positive cells (Supplementary Figure 5).

Similarly, hiPSC were also able to differentiate into ECs when cultured under specific conditions. RT-PCR and immunostaining have been performed at day 25 after differentiation start. Three different clones differentiated into ECs showed high expression of the ECs markers CD31, von Willebrand factor (vWF) and endoglin and concomitantly downregulation of the pluripotency markers OCT4, NANOG, and REX1 (Figure 2C and 2D, Supplementary Figure 6). Moreover, hiPSC-derived ECs expressed markers including CD31, vWF and endothelial nitric oxide synthase (eNOS) comparable to human umbilical cord vein-derived endothelial cells (Figure 2C). The differentiation efficiency based on CD31 flow cytometric analysis showed a variability between 52.7% and 84.2% (Supplementary Figure 7). In conclusion, the obtained hiPSC-derived SMCs and ECs did not show expression of any of the pluripotency markers by immunostaining and PCR, and resembled native SMCs and ECs based on their characteristic markers. In total 3 clones were differentiated into SMCs or ECs. After differentiation, the three clones showed a similar expression profile at RNA and protein level between each other (Supplementary Figure 3 and 6).

3.3 Qualitative and quantitative tissue analysis of TEVGs based on human iPSCs

Human iPSC-derived SMCs and ECs were seeded onto a PGA/P4HB scaffold and cultured under static or dynamic conditions. Microstructural analysis using scanning electron microscopy (SEM) was used to analyze the fiber size and porosity of unseeded polymers before and after coating (Supplementary Figure 8). Quantitative measurements of the fiber diameter and porosity (n=4 per condition) were conducted. The fiber diameter was uniform among all biomaterials with no statistically significant differences between the hybrid polymer or uncoated PGA. PGA-P4HB scaffolds were significantly less porous than PGA ($p<0.05$) (Supplementary Figure 8f). After 3, 6 and 9 weeks the constructs were retrieved for histological analysis (Figure 4). Hematoxylin and Eosin (H&E) staining demonstrated formation of substantial amounts of extracellular matrix (ECM) *in vitro* and Masson Trichrome (MT) staining confirmed deposition of collagen fibers. Nevertheless, TEVGs under static conditions displayed high cellularity and layered tissue on the outer vessel regions, while in the central part of the scaffold low cellularity and no visible formation of ECM were present (Figure 3, rows 1, 3 and 5). In contrast, TEVGs under dynamic conditions exhibited high cellularity and tissue formation on the outer as well as on the inner vessel regions (Figure 3, rows 2, 4 and 6). Nonetheless, low cellularity was demonstrated between these two layers under static conditions compared to native controls after 3 weeks of culture. Generally, under dynamic culture the cells distributed homogenously within the scaffolds and displayed a better collagenous ECM deposition on the outer as well as on the inner vessel regions in comparison to TEVGs under static conditions (Figure 3). In sum, vascular grafts cultured under dynamic conditions showed a better cellular infiltration and tissue formation in comparison to static culture. Furthermore, dynamically cultured TEVG (n=4) show a significant increase of wall thickness under dynamic conditions in comparison to statically cultured TEVG over culture time ($p<0.0001$, Supplementary Figure 10).

In order to quantify the composition of ECM, TEVGs were biochemically analyzed using assays for hydroxyproline (HYP), glycosaminoglycans (GAG), and the cell number

(deoxyribonucleic acid, DNA) (Figure 4A). These findings underlined the histological observations showing cell proliferation and increasing ECM formation over the analyzed 9 weeks period. Moreover, TEVGs generated with the three hiPSC clones displayed similar amounts of HYP, GAG and DNA for the respective conditions with no detectable significant differences, indicating lack of inter-individual differences (Supplementary Figure 9).

Overall dynamic conditions (n=4) showed enhanced cell proliferation ($p<0.05$) and significant higher expression of GAG ($p<0.0001$), and HYP ($p<0.0001$) in comparison to static conditions after 9 weeks of culture. Interestingly, the amount of DNA ($p<0.0001$), GAG ($p<0.0001$), and HYP ($p<0.03$) is also significant higher between 3 weeks and 9 weeks under dynamic conditions. Polarization microscopy revealed the presence of initial scaffold matrix in the central part of the constructs (Figure 4B). However, semi-quantitative analysis of dynamic constructs showed that the biomaterial remnants were significant more degraded ($p<0.001$) compared to static constructs after 9 weeks of culture. These findings additionally underline the histological observations previously described. The co-polymer starter matrices showed less remodeling and scaffold degradation in the central part of the constructs under static as well as dynamic conditions given the lack of tissue formation in this area of the constructs.

3.4 Immunofluorescence of TEVGs

To determine the cellular phenotype in the TEVGs derived from hiPSCs, cells were stained for αSMA and vWF. Immunofluorescence staining of TEVGs under static as well as dynamic conditions demonstrated layered tissue architecture with an αSMA positive layer located in the interstitium and a thin luminal layer of vWF positive cells comparable to native vessels (Figure 5). After 3 weeks of culture the αSMA positive layer of TEVGs were thinner in comparison to native tissue. The majority of cells stained positive for αSMA were located at the outer layer of the statically cultured TEVGs. In contrast, dynamically cultured TEVGs showed a thicker αSMA positive layer (Figure 5).

4 Discussion

In vitro engineering of small diameter native-like vascular grafts is still demanding as poor haemocompatibility of TEVGs often leads to thrombosis and stenosis when implanted *in vivo* [5]. Therefore, creating a functional biomaterial that closely mimics the native layered architecture of blood vessels would represent a major step towards the functional replacement of diseased vascular structures. The current study is the first report showing the possibility to employ human PBMC-derived iPSCs to generate synthetic scaffold-based TEVGs, which contained a αSMA-positive layer in the graft interstitium as well as a thin luminal layer of vWF-positive cells comparable to native vessels. Importantly, given the ease of access, this PBMC-based approach may open up the possibility to generate native-like endothelialized 'customized' TEVGs to study complex human diseases *in vitro* not only enabling better understanding of the pathophysiology, but ultimately also allowing for assessment of novel therapeutic approaches in the future.

Our data highlight the possibility to reprogram PBMCs into hiPSCs and make use of these cells for engineering small diameter vascular grafts. The use of PBMCs has clear advantages over dermal fibroblasts, which represent the most common source to generate hiPSCs [19, 24]. For example, exposure of the dermis to ultraviolet (UV) light increases the risk for chromosomal aberrations [25], further fibroblast cells need to expand for several passages *in vitro* and make it a cumbersome cell source for reprogramming. In addition, taking skin biopsies usually results in scar tissue formation and is less practical for non-therapeutic disease modeling settings or in volunteers. Beyond that, skin represents a non-reconstituting organ not providing unlimited supply to a certain donor tissue, implying that repetitive cell harvest seems more difficult. In contrast, peripheral blood is a constantly reconstituting source of patient tissue that is not exposed to UV radiations. Moreover, it represents a low invasive and well-established cell source – a cell source one could use for large scale with repetitive isolation of sterile, viable, autologous, cellular material from patients as well as from voluntary donors.

Notwithstanding, direct reprogramming of somatic cells into cells of clinical value provides also a promising alternative strategy. The main advantages are (i) reduced time consumption, (ii) limited costs, and (iii) low risk for tumor formation. However, the reprogramming efficiency and the limited scalability of this cell generation system are the key challenges. In addition, the different cell types for direct reprogramming into SMCs and ECs in both mice and humans are nowadays limited to fibroblasts and amniotic fluid derived cells [26].

Various reprogramming techniques have been established during the last years for generating hiPSCs [27]. So far, four main categories have been subdivided: integration-defective viral delivery, episomal delivery, RNA delivery and protein delivery [27]. The original method of reprogramming murine fibroblasts by Yamanaka utilized retroviral transduction of Oct4, Sox2, Klf4, and c-Myc into MEF [19]. As lentiviruses, unlike retroviruses, can infect non-dividing and proliferating cells, this approach became the most preferred reprogramming procedure.

In particular, for clinical applications reducing genomic modifications to prevent insertional mutagenesis is mandatory. Therefore, in order to obtain cells suitable for clinical application, transgene-free hiPSCs need to be employed to avoid transgene reactivation, altered gene expression and misguided differentiation. For this purpose, we and other laboratories studied the possibility of developing more and more non-integrative or semi-integrative reprogramming approaches [28].

Another critical factor influencing the generation of TEVGs are the conditions in which they are cultured. We analyzed hiPSC-derived TEVGs cultured under static and dynamic conditions and could demonstrated in the dynamic cultures increased cellularity, more tissue formation and scaffold degradation when compared to static culture, as confirmed previously [29]. It has been shown that mechanobiological interactions between cells and scaffolds can crucially influence cell behavior [29, 30]. Wang et al. also showed an enhanced cell proliferation under dynamic three-dimensional (3D) culture compared with conventional static two-dimensional (2D) and 3D cell culture conditions [31]. Niklason and colleagues

464 investigated the effects of 8 weeks cyclic mechanical loading on tubular meshes of PGA
465 seeded with adult bovine aortic smooth muscle cells placed around distensible silicone tubes
466 [32]. The so-generated TE arteries were then implanted in miniature swine and showed a
467 patency up to 24 days.
468 The long-term patency of small-diameter vascular grafts is still a great challenge in the field
469 of cardiovascular TE research. Early luminal thrombosis represents the major cause of
470 vessel occlusion. A possible solution represents the coating of the grafts with
471 antithrombogenic materials, such as heparin [33] or ethylene oxide [34], but the results still
472 could not overcome the limitation completely. The vascular TE approach has been
473 introduced to generate vascular grafts with functional endothelium potentially enabling long-
474 term patency [35]. This project showed the production of hiPSC-derived TEVGs, which
475 contained a αSMA-positive layer in the interstitium and a thin luminal layer of vWF-positive
476 cells comparable to native vessels. This underlines the impact of a functional endothelial cell
477 layer in TEVGs. Future efforts will focus on the implantation of our TEVGs also to investigate
478 the *in vivo* functionality and patency of human PBMC-iPSC-based TEVGs compared to
479 mature vascular cell-derived constructs.
480 In the past, murine [36] and hiPSCs [37] were differentiated into SMCs and used to construct
481 a TEVG for subcutaneous implantation for 2 weeks. Furthermore, Gui et al. implanted a
482 hiPSC-derived TEVG into nude rats as abdominal aorta interposition for 2 weeks [38]. These
483 TEVG contained αSMA and SMMHC-positive cells. Nevertheless, they also observed
484 thrombus formation in some samples, possibly due to the lack of endothelial cell coating of
485 vascular grafts before implantation [32]. So far, these studies have been based on the
486 reprogramming of human fibroblasts. Recently, Hu et al. established integration-free
487 reprogrammed hiPSCs from PBMCs and then differentiated the cells into mesoderm-
488 originated cardiovascular progenitor cells (CVPCs) [39]. Subcutaneous implantation of
489 CVPCs seeded on a disc shaped scaffold led to *in vivo* lineage specification [39]. However,
490 no fully autologous TEVG based on SMCs and endothelial cells (ECs) differentiated from
491 PBMC-derived hiPSC based on a fully biodegradable scaffold system has been realized so

far. Our data demonstrates for the first time the feasibility to use pre-differentiated human ECs and SMCs differentiated from PBMC-derived hiPSCs for the production of bioengineered blood vessels with architecture comparable to native vessels.

5 Conclusion

Human iPSCs generated from PBMCs is a promising cell source for TE as they are easily accessible and may thus serve as a versatile platform also involving disease-modeling applications. In addition to solving ethical concerns related to the use of blastocyst-derived ESCs, the use of hiPSCs for the generation of therapeutic cells may avoid the necessity for post-transplant immunosuppression. Upon appropriate differentiation, these cells can then be used to study normal and pathologic human tissue development *in vitro*, enabling new insights into disease pathology as well as the development of novel therapeutic agents and patient-specific cellularized replacements. In summary, our study established an efficient approach towards generating patient-specific small diameter TEVGs based on human PBMC-iPSC-derived SMCs and ECs, containing native-like layered vessel architecture.

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Competing Interests

The authors declare no competing non-financial and financial interests.

Contributions

M.G. performed and designed the experiments, collected and analyzed the data, and wrote the manuscript with significant editorial contribution from P.C., B.W., and E.A.C.. E.A.C. and P.C supervised in designing the experiments, collecting and interpreting the data. D.K. helped in collecting and analyzing data. D.W. helped in collecting data. B.W., and S.P.H. conceived the project.

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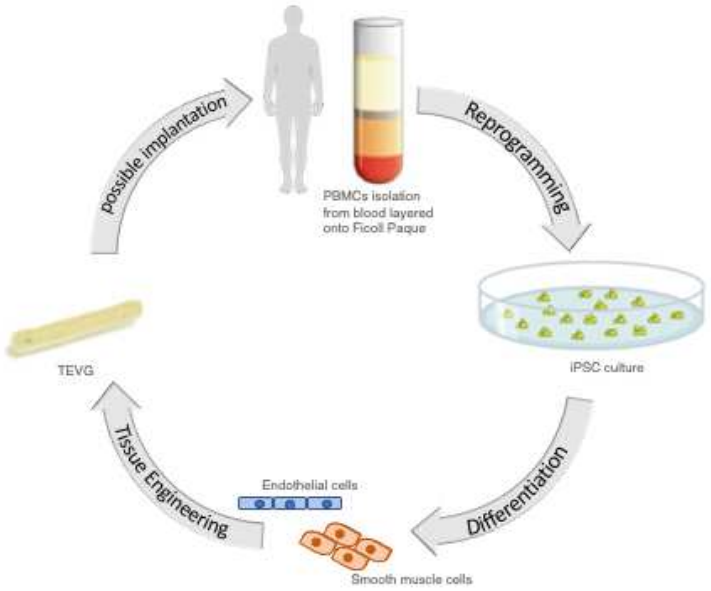
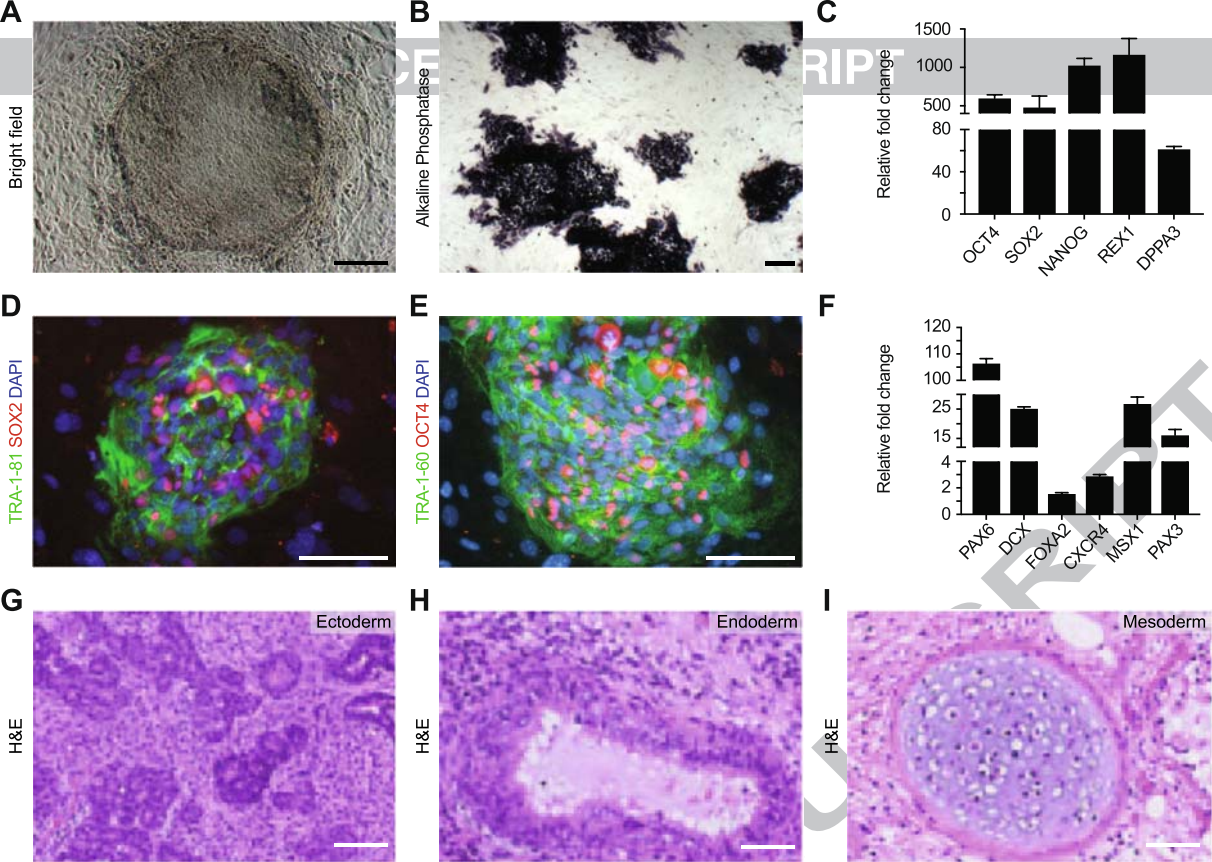
Statement of Significance

We report for the first time the possibility to employ human peripheral blood mononuclear cell (PBMC)-derived iPSCs to generate biodegradable polymer-based tissue engineered vascular grafts (TEVG), which mimic the native layered architecture of blood vessels.

hiPSCs from PBMCs were differentiated into smooth muscle cells as well as endothelial cells. These cells were co-seeded on a biodegradable PGA/P4HB scaffold and cultured in a bioreactor to induce tissue formation *in vitro*. The resulting small diameter TEVG showed abundant amounts of extracellular matrix, containing a αSMA-positive layer in the interstitium and a thin luminal layer of vWF-positive endothelial cells approximating the architecture of native vessels.

Our findings improving the generation of autologous vascular replacements using blood as an easily accessible cell source.

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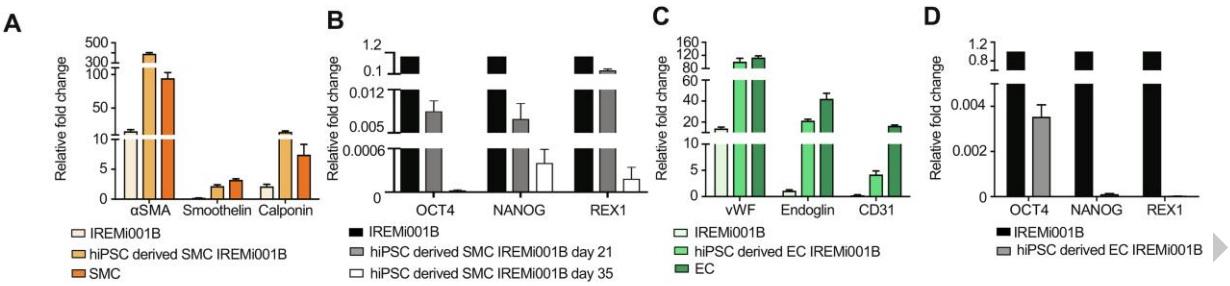

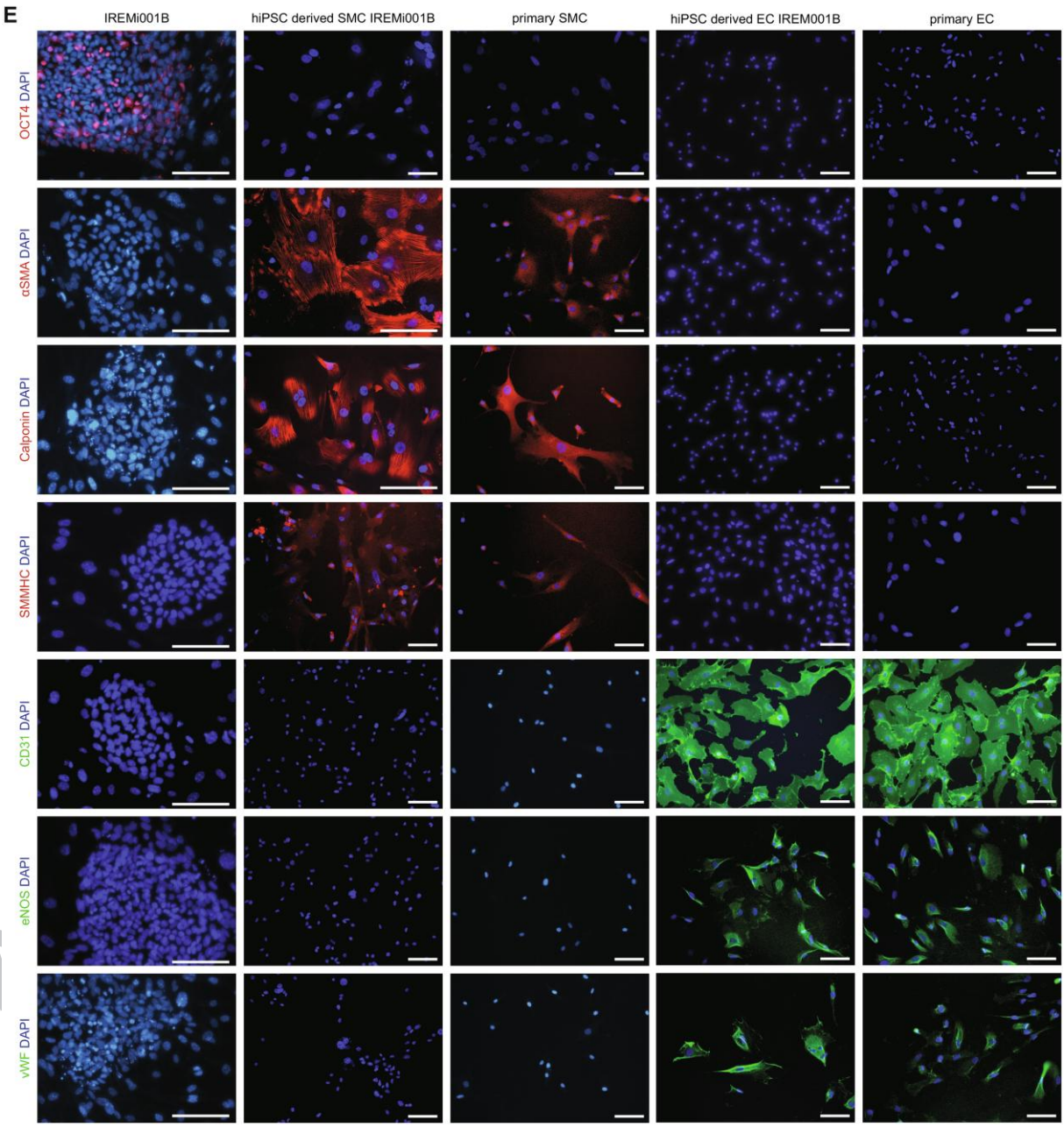

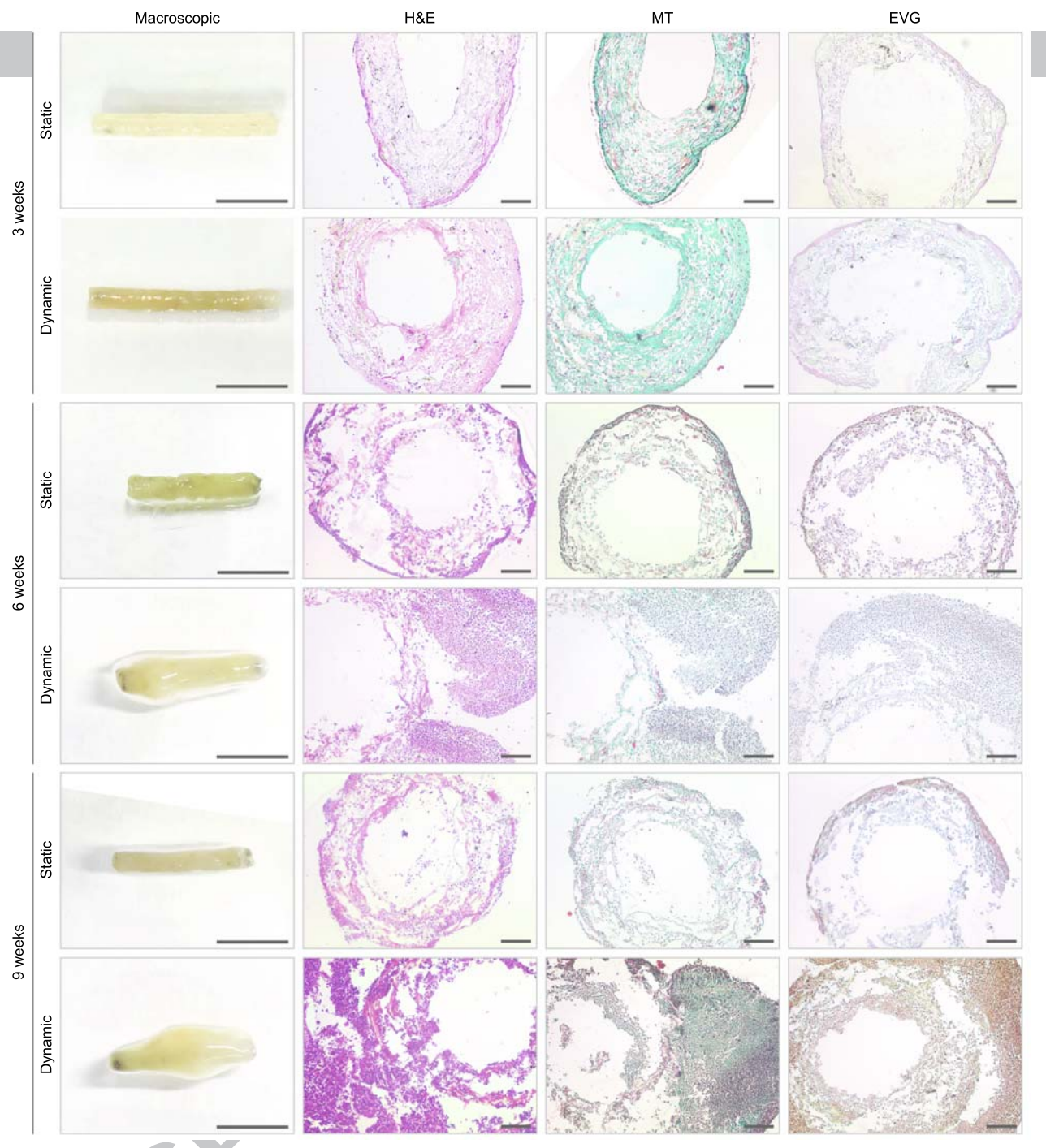
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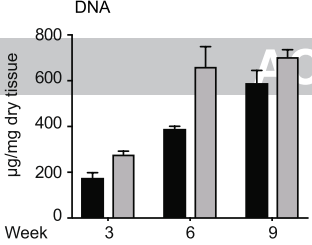

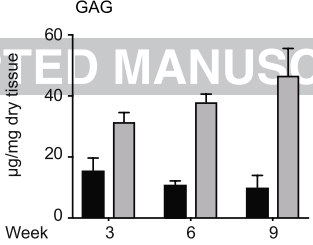

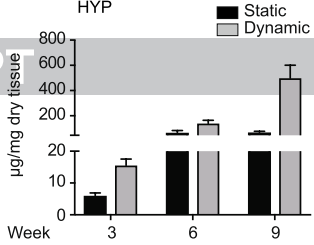

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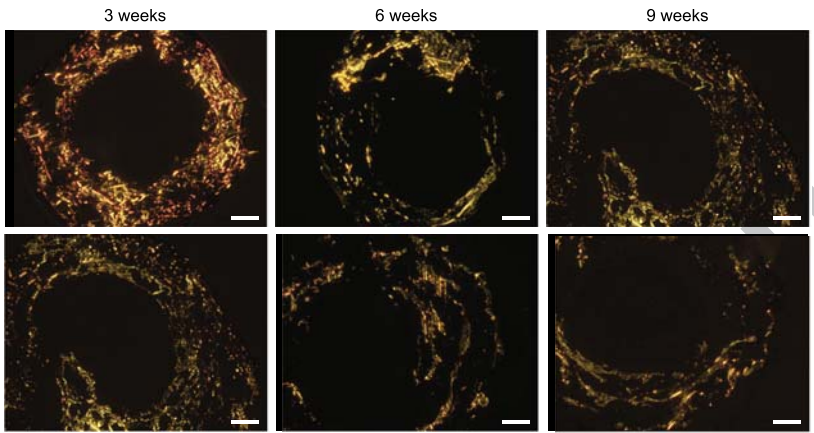

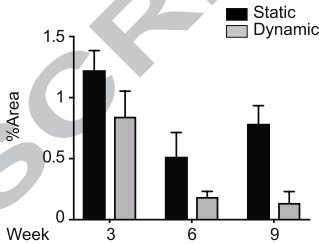

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